Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology and Applied Pharmacology 207 (2005) S161 – S170
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Review

Assessment of hepatotoxic liabilities by transcript profiling

Stefan Ruepp[a,*], Franziska Boess[a], Laura Suter[a], Maria Cristina de Vera[a], Guido Steiner[b], Thomas Steele[c], Thomas Weiser[a], Silvio Albertini[a]

[a]F. Hoffmann-La Roche Ltd., PRBN-S (90/5.18), CH-4070 Basel, Switzerland
[b]Bioinformatics, 4 CH-4070 Basel, Switzerland
[c]Non-Clinical Drug Safety, Nutley, NJ, USA

Received 15 July 2004; revised 25 January 2005; accepted 31 January 2005
Available online 28 June 2005

Abstract

Male Wistar rats were treated with various model compounds or the appropriate vehicle controls in order to create a reference database for toxicogenomics assessment of novel compounds. Hepatotoxic compounds in the database were either known hepatotoxicants or showed hepatotoxicity during preclinical testing. Histopathology and clinical chemistry data were used to anchor the transcript profiles to an established endpoint (steatosis, cholestasis, direct acting, peroxisomal proliferation or nontoxic/control). These reference data were analyzed using a supervised learning method (support vector machines, SVM) to generate classification rules. This predictive model was subsequently used to assess compounds with regard to a potential hepatotoxic liability. A steatotic and a non-hepatotoxic $5HT_6$ receptor antagonist compound from the same series were successfully discriminated by this toxicogenomics model. Additionally, an example is shown where a hepatotoxic liability was correctly recognized in the absence of pathological findings. In vitro experiments and a dog study confirmed the correctness of the toxicogenomics alert. Another interesting observation was that transcript profiles indicate toxicologically relevant changes at an earlier timepoint than routinely used methods. Together, these results support the useful application of toxicogenomics in raising alerts for adverse effects and generating mechanistic hypotheses that can be followed up by confirmatory experiments.

Keywords: Toxicogenomics; Microarray; Rat; Liver

Contents

Introduction

Drug-induced liver toxicity is a common cause of liver injury resulting in drug withdrawal from the market or

* Corresponding author. Fax: +41 61 688 8101.
E-mail address: stefan.ruepp@roche.com (S. Ruepp).

0041-008X/$ - see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.taap.2005.05.008

severely restricted use. This encourages improvements in current pre-clinical and clinical testing. Traditionally, pre-clinical testing involves descriptive histopathological examination and measurement of serum enzyme levels to investigate the hepatic liability of a development compound. New approaches that improve upon conventional processes of risk assessment and safety evaluation are currently sought. High-density microarrays that allow simultaneous monitoring of transcriptional changes of thousands of genes in response to various stimuli are a promising new technology. The application of gene expression analysis in toxicology has led to the emergence of the discipline of toxicogenomics. Great hopes on the application of transcript profiling in toxicology justified significant investment in toxicogenomics efforts in most pharmaceutical companies as well as academic and governmental institutions. Toxicogenomics is anticipated to improve not only sensitivity and accuracy, but also speed of toxicological investigations. In addition, determination of potential liabilities of compounds early in the drug development process can save development time and money by focusing resources on compounds that are more likely to succeed (Waring and Ulrich, 2000).

Some of the earliest success stories of microarray based classification described the transcriptional differences in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (McClelland et al., 2001). This discrimination is critical for successful treatment (Golub et al., 1999). Another work described the molecular characterization of 60 cell lines from diverse tumor tissues indicating improved sensitivity of the microarray approach compared to traditional histopathology (Ross et al., 2000). A further study investigated transcriptional effects of 118 cancer drugs with known mechanisms of action in these 60 tumor cell lines. Transcript profiles associated with specific drug treatments showed a good correlation with the mechanism of action (Scherf et al., 2000) and represent remarkable reproducible features that allow the recognition of distinct groups. The same tumor subtype defined in one breast tumor data set could also be recognized in 2 independent breast tumor data sets. The transcriptional alterations were considered to be major determinants for disease outcome (Sorlie et al., 2003).

The underlying assumption of toxicogenomics is that toxicity is accompanied by transcriptional changes which are either causally linked to the mechanism of toxicity or represent a response to a toxic insult. Several publications linked toxicity to expression changes of individual genes or groups of genes (Hamadeh et al., 2002b; Ruepp et al., 2002; Suter et al., 2003). The predictive toxicogenomics approach assumes that similar treatments leading to the same toxic end-point will share comparable changes in gene expression. The potential of predictive toxicogenomics was highlighted by the identification of blinded samples using gene expression profiles from hepatotoxicants (Hamadeh et al., 2002a). Successful discrimination of gene expression fingerprints was also reported in several other studies (Bartosiewicz et al., 2001; Bulera et al., 2001; Thomas et al., 2001; Waring et al., 2001).

The liver is a primary site for drug metabolism and is frequently involved in adverse drug reactions. Therefore, we chose the liver as a target organ for the described toxicogenomics studies. A multitude of hepatotoxic as well as some non-hepatotoxic compounds and matched vehicle controls were administered to male Wistar rats. Liver gene expression profiles were subsequently used to create a reference database.

For all studied compounds, hepatotoxic mechanisms are well known and fall in one of the following categories. The microvesicular form of hepatic *steatosis* (fatty liver) is especially clinically relevant. Fatty liver is thought to occur due to mitochondrial damage causing impairment of β-oxidation and accumulation of small lipid vesicles within hepatocytes. A severe decrease in energy production may cause hepatic failure, coma and death (Pessayre et al., 1999). Failure of bile excretion is a pathophysiologic process termed *cholestasis*. Intra-hepatic cholestasis is often caused by inhibition of bile acid transporters that leads to accumulation of hepatocellular bile acids, resulting in liver injury, inflammation and elevated levels of circulating alkaline phosphatase (Jaeschke et al., 2002; Velayudham and Farrell, 2003). Damage to macromolecules such as proteins and lipids can be caused by direct interaction through a toxic compound itself or, more commonly, through a highly reactive metabolite (Lee et al., 1992). Pathophysiological manifestations of such *direct acting* compounds are hepatocellular necrosis, lipid peroxidation and elevated levels of circulating alanine-aminotransferase (ALT). Additionally, we investigated a series of peroxisome proliferator-activated receptor (PPAR) agonists which cause peroxisome proliferation. *Peroxisome proliferators* (PPs) are non-genotoxic rodent hepatocarcinogens that cause liver enlargement and hepatocarcinogenesis associated with peroxisome proliferation, induction of hepatocyte DNA synthesis and suppression of apoptosis (Roberts et al., 2000).

For predictive tasks, supervised methods have been described as promising tools (Baumgartner et al., 2004; Bullinger et al., 2004; Scholkopf and Smola, 2002). We used Support Vector Machines (SVMs), which belong to the class of supervised learning algorithms (Boser et al., 1992; Vapnik, 1998) and were reported to perform well in different areas of biological analysis (Scholkopf and Smola, 2002). Given a set of training examples, SVMs are able to recognize informative patterns in input data and make generalizations on previously unseen samples. Like other supervised methods, SVMs require prior knowledge of the classification problem, which has to be provided in the form of labeled training data. We used histopathological assessment and clinical chemistry data to allocate profiles to a specific training class. We combined the creation of a model with recursive feature elimination (RFE; (Guyon et al., 2002)), to identify the most discriminative gene changes. A model discriminating controls and non-hepatotoxic expres-

sion profiles from the four categories described above was created and used to assess novel gene expression profiles. Classification of RNA expression-patterns successfully discriminated between a hepatotoxic and a non-hepatotoxic serotonin receptor (5-HT) antagonist from the same chemical series. This predictive toxicogenomics approach also flagged a further development compound as potentially hepatotoxic, despite absence of histopathology in the rat. This alert was confirmed in in vitro experiments and in a dog study. Finally, some examples are provided indicating an earlier detection of toxicity, preceding pathology, using this toxicogenomic approach.

Materials and methods

Animal treatment. Permission for animal studies was obtained from the local regulatory agencies, and all study protocols were in compliance with animal welfare guidelines. Male HanBrl: Wistar rats approximately 12 weeks of age (300 g ± 20%) were obtained from BRL, Füllinsdorf, Switzerland. All rats were sacrificed by carbon dioxide (CO_2) asphyxiation, exsanguinated and necropsied after oral (gavage) treatment with the test compound. In all studies, a time-matched vehicle control group was included.

Rx65 and Rx66. Rats received a single dose p.o. (gavage) of Rx65 or Rx66 (400 mg/kg, vehicle distilled water) and were sacrificed 6 or 24 h after dosing. With Rx65 rats were also dosed sub-acute for 7 days at 30, 100 or 400 mg/kg/day and sacrificed 24 h after the last administration. All treatment groups consisted of 5 animals.

Rx8, Rx9 and Rx10. Rats were dosed with a single or repeated dose for 5 days p.o. (gavage) with Rx08, Rx09 or Rx 10 dissolved in klucel and sacrificed after 24 h. Dose amounts were 250 mg/kg/day for Rx08, 100 mg/kg/day for Rx09 and 125 and 500 mg/kg/day for Rx10. Within a treatment group all six animals were dosed equally, but 3 rats were used for histopathological assessment of the livers while livers from the remaining 3 animals were used for additional measurements including gene expression profiling.

Coumarin, 6-methylcoumarin and tacrine. Rats were dosed p.o. (gavage) with 200 mg/kg coumarin or 6-methylcoumarin dissolved in corn oil and assessed after 6 or 24 h. Rats were dosed i.p. with 35 mg/kg tacrine, dissolved in 7.5% gelatine and assessed after 6 or 24 h.

Rx10, Beagle dogs. Beagle dogs aged 16 months (Marshall Farms, USA) were housed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and sacrificed with an overdose of sodium pentobarbital after treatment with the test compound. Two male and 2 female dogs were treated for 14 days with an escalating dose of Rx10 (15, 30, 60, 120 mg/kg/day) p.o. (gavage).

Hepatocyte preparation, culture and treatment. Hepatocytes were isolated from 10- to 14-week-old naive male HanBrl:WIST rats by a two-step collagenase liver perfusion method (Berry and Friend, 1969) as previously described (Goldlin and Boelsterli, 1991). Briefly, the rats were anaesthetized with sodium pentobarbital (120 mg/kg, i.p.). The liver was first perfused for 5 min with a pre-perfusing solution consisting of calcium-free, EGTA (0.5 mM)-supplemented, HEPES (20 mM)-buffered Hank's balanced salt solution (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.55 mM D-glucose). This was followed by a 12-min perfusion with $NaHCO_3$ (25 mM)-supplemented Hank's solution containing $CaCl_2$ (5 mM) and collagenase (0.2 U/ml). Flow rate was maintained at 28 ml/min and all solutions were kept at 37 °C. After in situ perfusion the liver was excised and the liver capsule was mechanically disrupted. The cells were suspended in William's Medium E without phenol red (WME) and filtered through a set of tissue sieves (30-, 50- and 80-mesh). Dead cells were removed by a sedimentation step (1 × *g*, for 15 min at 4 °C) followed by a Percoll-centrifugation step (Percoll density: 1.06 g/ml, 50g, 10 min) and an additional centrifugation in WME (50 × *g*, 3 min). Typically, $100-300 \times 10^6$ cells were obtained from one rat liver. Hepatocyte viability was assessed by trypan blue exclusion and typically ranged between 85% and 95%. Cells were seeded into collagen-coated 6-well Falcon Primaria plates (Fisher Scientific AG, Wohlen, Switzerland), at a density of 9×10^5 cells/well in 2 ml WME supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (100 nM) and dexamethasone (100 nM). After an attachment period of 3 h, the medium was replaced by 1.5 ml/well serum-free WME, supplemented with antibiotics and hormones, and further kept at 37 °C in an atmosphere of 5% CO_2/95% air. After a further overnight pre-culture period, the cells were exposed to the test compound dissolved in WME (final concentration of DMSO vehicle 0.2%) or to the corresponding vehicle (WME containing 0.2% DMSO).

Cytotoxicity assessment—LDH-release. Acute cytotoxicity was determined as lactate dehydrogenase (LDH) release into the cell culture medium. LDH activity was determined spectrophotometrically using commercially available test kits (Roche Diagnostics, Mannheim, Germany) on a Cobas Fara autoanalyzer (Roche, Rotkreuz, Switzerland). Enzyme activity in the medium was expressed as percentage of total LDH activity present in the cells at the beginning of the incubation.

Fatty acid β-oxidation. The formation of acid-soluble products from [U-^{14}C]-palmitic acid was determined as a measure for β-oxidation. The cells were incubated with 1 mM palmitic acid containing tracer amounts of [^{14}C]-palmitic acid in the presence or absence of test compound (medium was Williams medium E supplemented with

insulin and 2% BSA) and in the presence or absence of exogenously added carnitine (500 μM). Medium samples (250 μl) were collected after 2 h and acidified by adding perchloric acid. Subsequently, KOH was added and proteins were precipitated by centrifugation. The radioactivity from short-chain β-oxidation products was then measured in the supernatant in a Beckman LS 6000LL liquid scintillation analyzer and expressed as nmol palmitic acid equivalents. The carnitine-sensitive palmitic acid β-oxidation, indicative of the mitochondrial contribution to β-oxidation, was then calculated as difference of short-chain β-oxidation products formation in the presence and absence of exogenously added carnitine. The results were expressed as percentage of carnitine sensitive palmitic acid β-oxidation in vehicle treated control cells.

Measurement of cellular lipid accumulation. Accumulation of lipophilic inclusions was assessed using nile red staining (Fowler et al., 1987; Greenspan et al., 1985). Nile red is a fluorescent dye which accumulates and fluoresces in lipophilic environment, while fluorescence is quenched in water. The cells were incubated for 10 min at 37 °C, with the staining solution (10 μg/ml nile red in PBS). After removing the staining solution, the fluorescence from the cell layer was then determined in a Victor^2 multilabel counter (EG and G Wallac, Regensdorf, Switzerland). The results were expressed as percentage of the control.

Measurement of triglycerides. After treatment of cells for the desired time period, the medium was aspirated and the cells were washed with ice-cold saline, harvested in a total volume of 0.5 ml saline and homogenized by ultrasonication. Aliquots were taken from the homogenate to determine total triglycerides concentration. Triglyceride concentration was determined with a test kit and by means of a Cobas/Fara autoanalyzer (Roche Diagnostics AG, Basel, Switzerland).

Histopathology. Representative liver samples were fixed in 10% neutral buffered formalin. One additional liver sample from the cranial half of the left lateral lobe was placed in Carnoy fixative for glycogen staining. All samples were processed using routine procedures and embedded in Paraplast. Tissue sections approximately 2–3 μm were cut and stained with hematoxylin–eosin (HE) or periodic acid-Schiff (PAS) for glycogen. Fat Red 7B stain was performed on frozen formalin-fixed sections in order to visualize lipid deposits. Microscopic findings were evaluated for severity using a five-point grading scale: 1 (minimal), 2 (slight), 3 (moderate), 4 (marked), 5 (severe).

Sample preparation and hybridization. RNA isolation, processing and hybridization were essentially carried out as recommended by Affymetrix (www.affymetrix.com, Affymetrix, Santa Clara, CA) with minor modifications (Steiner et al., 2004).

Data acquisition and preprocessing. Primary data were obtained by laser scanning (Hewlett Packard, Palo Alto, CA, USA) and collated using the Affymetrix Microarray Suite Version 5.0 software (Affymetrix, Santa Clara, CA). Before doing any downstream analysis, data were preprocessed in a standardized way (details in (Steiner et al., 2004)).

Support vector machines. Details concerning theory and application of support vector machines are described in the literature (Cristianini and Shawe-Taylor, 2000; Scholkopf and Smola, 2002). All SVM classifications were based on the freely available software package libsvm 2.36 (Chang and Lin, 2001). The source code was extended to meet our needs and compiled to run on SGI IRIX 6.5. Extensions such as parameter optimization, recursive feature selection, enhanced cross validation (CV) options, one-versus-all training scheme and report generation were implemented in a C library on top of libsvm. The SVM was calculated as described (Steiner et al., 2004) with the minor modification that aflatoxin and phalloidin were removed from the cholestatic training classes and lithocholic acid was added to this class. The reason for this a certain overlap of cholestatic and "direct acting" properties of the former 2 compounds.

A linear kernel $k(x_i,x_j) = \langle x_i,x_j \rangle$ was chosen for the SVM. In order to handle the multiple class situation, we applied the one-versus-one (OVO) training paradigm. Following this approach, a set of binary SVMs is created each of which separates the samples of one class (positive examples) from another training class (negative examples). A voting scheme of the individual SVMs is subsequently applied to determine the classification of a given sample.

Results

Generation of a predictive model using a reference database

We created a reference database with liver gene expression profiles from vehicle and compound treated rats. Subsequently, SVMs were used as a supervised learning method to generate classification rules. Histopathology and clinical chemistry results in conjunction with published data provided a rational basis for allocating individual gene expression profiles of rat livers treated with a variety of compounds to the 5 training classes: controls, direct acting, cholestasis, steatosis and peroxisomal proliferators. (Direct acting: bromobenzene, CCl_4, hydrazine, thioacetamide, 1,2-dichlorobenzene, coumarin, acetaminophen; Steatosis: amineptine, amiodarone, 4 proprietary compounds; Cholestasis: chlorpromazine, cyclosporin A, glibenclamide, lithocholic acid, methylene dianiline; Peroxisomal Proliferation: WY-14V643, 5 proprietary compounds; Controls: 163 time-matched vehicle control rats. Each treatment group usually consisted of 5 animals.). A detailed description of the

compounds including histopathology and clinical chemistry is described in Steiner et al. (2004). The model described therein was slightly modified for the current study. Aflatoxin and Phalloidin were omitted from the training class since both compounds had overlapping "direct acting" and cholestatic effects and lithocholic acid was additionally included in the cholestatic model. The classification approach was one-versus-one (e.g., cholestasis vs. control, cholestasis vs. steatosis etc.). The SVM used a linear kernel and validation was performed in a compound-based external cross-validation (CV) procedure. This means that all profiles from a given compound were omitted from SVM model building including feature ("gene") selection. Compound induced expression profiles were classified by an SVM where these specific compound profiles were omitted from training.

Gene expression based discrimination of the hepatotoxic liability of two 5HT$_6$ compounds

Two 5-HT$_6$ receptor antagonists were investigated in male rats. Both compounds share the same pharmacological target, but have remarkably different toxicity profiles. Rats were dosed with a single dose (400 mg/kg) Rx65 or Rx66 and gene expression changes were assessed after 6 or 24 h. In a repeated dose study (7 days) Rx65 was administered at 30, 100 or 400 mg/kg/day. Gene expression profiles were assessed in a blinded manner by the previously described SVM. Already after 24 h, a clear discrimination of both compounds was possible by this computational approach. Whereas Rx66 expression profiles were not different from control gene expression profiles, Rx65 profiles were identified as steatotic. In the subchronic dosing scheme (1 week, daily administration), the steatosis produced by 400 mg/kg Rx65 was corroborated with the SVM. With the mid-dose (100 mg/kg) 3 out of 4 animals were classified as steatotic, whereas the lowest dose was predominantly classified as controls (Table 1).

Histopathology confirmed a dose-related increase in fatty change in the liver observed after repeated dosing with Rx65.

Table 1
SVM predictions for all groups that were treated with the 5-HT$_6$ receptor antagonists Rx65 and Rx66

Compound	Dose	Treatment	Time	SVM prediction
Rx65	400 mg/kg/day	Repeated dose	168 h	Steatotic
Rx65	100 mg/kg/day	Repeated dose	168 h	Steatotic (75%) Control (25%)
Rx65	30 mg/kg/day	Repeated dose	168 h	Control (80%) Steatotic (20%)
Rx65	400 mg/kg/day	Single dose	24 h	Steatotic
Rx66	400 mg/kg/day	Single dose	24 h	Controls
Rx65	400 mg/kg/day	Single dose	6 h	Controls
Rx66	400 mg/kg/day	Single dose	6 h	Controls

If classification results are not fully concordant within one group, percentage numbers are indicated.

Hepatocellular vacuolation, characterized by multiple small discrete periacinar vacuoles that sometimes coalesced to form larger ones (microsteatosis), was seen on HE-stained sections. These vacuoles stained positive for lipid. After 1 week of administration of Rx65, the mean severity of fatty change as assessed from Sudan-stained frozen sections increased from 1.0 in the low-dose group to 1.8 in the mid-dose group to 2.8 in the high-dose group. Only 1 out of 4 animals in the time-matched control group showed fatty change, while all rats treated with Rx65 for 7 days were affected. A single administration of Rx65 at 400 mg/kg resulted in an increase in the severity of fatty change (mean grade 2.2) at 24 h with respect to the controls (mean grade 1.2) and with Rx66 at 400 mg/kg (mean grade 1.4). The livers of rats 6h after treatment with Rx65 or Rx66 were comparable with their respective controls. The bioinformatics prediction based on liver transcript profiling confirmed the steatotic liability of Rx65 and absence thereof for Rx66.

Gene expression analysis based identification of a potential hepatotoxic liability of several development compounds

Male Wistar rats were treated with 3 different compounds of the same chemical class of antidiabetic compounds (Rx08, Rx09, Rx10). Rats were treated once or during 5 days with Rx08 (250 mg/kg/day), Rx09 (100 mg/kg/day) or Rx10 (125 or 500 mg/kg/day). The doses for Rx09 and the low dose of Rx10 were selected to result in similar efficacy, whereas Rx08 was a pharmacologically inactive stereo-isomer of Rx09, which was therefore applied at a dose level resulting in a similar plasma exposure as Rx09. Liver gene expression in individual animals was measured and subsequently assessed by the previously described SVM-based predictive toxicogenomics model (Table 2). The results suggest that all 3 compounds have similar transcriptional effects and have a steatotic liability in the rat liver. Repeated dosing with Rx08, Rx09 or Rx10 (500 or 125 mg) led to a unanimous classification as steatotic. After a single dose, the 2 former compounds could not be discriminated from controls by the present model although there were some transcriptional changes relative to the time-matched controls. However, the third substance was already flagged as steatotic at the high dose after single application. Hence, all substances are expected to have a significant steatotic potential in the rat liver.

While transcript profiles were determined in livers from 3 animals histopathology was performed on the remaining animals within each treatment group. Therefore, gene expression and pathology cannot be compared on an individual basis, but only on a group basis. In this combined study there was also some microvacuolation noted in control animals which complicates the task of identifying treatment-related changes. After treatment for 1 day only Rx10 did not produce microvacuolation, but Rx08, Rx09 and vehicle control did. Repeated dosing produced a similar picture: Oil-Red O-positive microvacuolation was noted in one

Table 2
SVM predictions for the treatment with antidiabetic compounds Rx08, Rx09 and Rx10

Compound	Dose	Single dose time	SVM prediction	Rep. dose time	SVM prediction
Rx08	250 mg/kg/day	24 h	Control	120 h	Steatotic
Rx09	100 mg/kg/day	24 h	Control	120 h	Steatotic
Rx10	500 mg/kg/day	24 h	Steatotic (67%) Cholestatic (33%)	120 h	Steatotic
Rx10	125 mg/kg/day	24 h	Control (67%) Cholestatic (33%)	120 h	Steatotic

Classification results are reported separately for the single dose (4th column) and repeated dose (6th column).

animal treated with Rx10, in 2 animals treated with vehicle or Rx08 and in all 3 animals treated with Rx09. However, several additional rat studies produced a clear result and established that Rx08 and Rx09, but not Rx10 were steatotic.

These discordant results with the 2 approaches led to subsequent in vitro analysis of all 3 compounds. Primary rat hepatocytes were treated in vitro with equimolar concentrations of the 3 compounds. Rx08 and Rx09 showed signs of cytotoxicity as measured by LDH release already with 150 μM, whereas Rx10 did not show cytotoxicity up to 300 μM. Intracellular lipid content of primary rat hepatocytes was measured using nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one). The non-cytotoxic concentrations 37.5 and 75 μM produced a dose-dependent increase in intracellular lipid content with all 3 compounds after 24 h incubation time (Fig. 1). The increase in intracellular lipid content was similar with all 3 compounds.

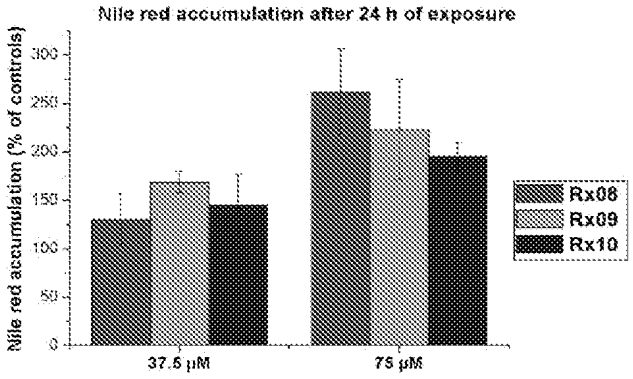

Fig. 1. Intracellular lipid content of primary rat hepatocytes treated for 24 h with non-cytotoxic concentrations (37.5 and 75 μM) of Rx08, Rx09 and Rx10 was measured using nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one). A similar dose-dependent increase in intracellular lipid content was observed with all 3 compounds. Error bars indicate the standard deviation derived from 3 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

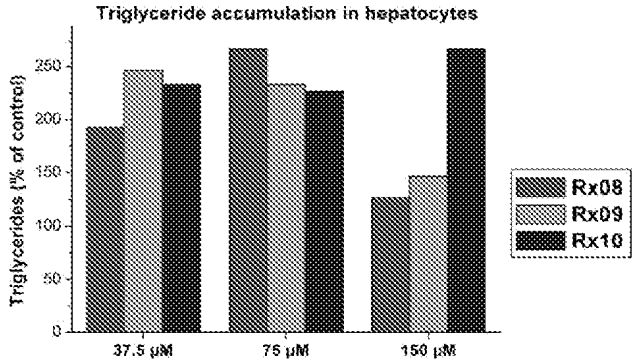

Fig. 2. Following a 24-h treatment period with Rx08, Rx09 or Rx10 hepatocellular triglyceride content was significantly increased with all 3 compounds. This increase was comparable at 37.5 and 75 μM. The 150-μM concentration of Rx08 and Rx09 was cytotoxic as determined by LDH release therefore the relative decrease in triglyceride content was probably caused by a reduced cell number. At this dose Rx10 caused a 2.5-fold elevation in triglyceride content in the absence of cytotoxicity.

Following a 24-h treatment period with Rx08, Rx09 or Rx10 hepatocellular triglyceride content was significantly increased (Fig. 2). At 37.5 and 75 μM, all 3 compounds led to a comparable and significant increase in triglycerides. At the highest dose (150 μM), Rx08 and Rx09 were already cytotoxic, therefore, the relative decrease in triglyceride content was likely to be caused by a reduced cell number per well. With Rx10 at this concentration, triglyceride content was more than 2.5-fold elevated in the absence of cytotoxicity. Thus, the observed increase in lipid content was primarily due to an accumulation of triglycerides in hepatocytes.

In order to test if this increase in triglyceride content was linked to impaired fatty acid catabolism, carnitine-sensitive β-oxidation was measured. Inhibition of β-oxidation could be confirmed with Rx09 and Rx10, but not with Rx08 at 75 and 150 μM (Fig. 3). However, at 300 μM, all 3 compounds showed a significant reduction in β-oxidation. The inhibition of β-oxidation was not caused by cytotoxicity as under

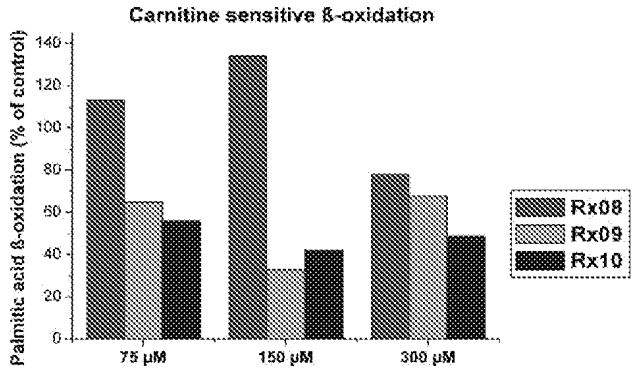

Fig. 3. Carnitine sensitive β-oxidation was measured to assess the effect on fatty acid catabolism. At 75 and 150 μM concentrations, β-oxidation was impaired following treatment with Rx09 and Rx10. At 300 μM, also Rx08 caused a significant reduction in β-oxidation. This inhibition of β-oxidation was not caused by cytotoxicity. The required presence of 2% albumin in the medium to dissolve palmitic acid abolished the cytotoxicity of Rx08 and Rx09 at this concentration.

the conditions for the β-oxidation assay, e.g., the presence of 2% albumin in the medium in order to dissolve palmitic acid, Rx09 and Rx10 were no longer cytotoxic at concentrations up to 300 μM.

In summary, this set of in vitro experiments confirmed the SVM based prediction on toxicogenomic data in terms of a steatotic liability for all 3 investigated compounds.

Since compound Rx10 was not hepatotoxic in rats according to conventional measures, the hepatotoxic potential was tested in a second species, namely the dog. Two male and 2 female beagle dogs were treated during 14 days with an escalating dose of Rx10 (p.o. (gavage) 15, 30, 60, 120 mg/kg/day). Histopathological assessment revealed increased incidence and severity of hepatotoxicity. Liver necrosis was observed in some animals at all doses, with severity increasing with dose. Interestingly, frozen liver sections stained for fat with Oil red-O confirmed also a steatotic liability. No vacuolation was observed with 15 mg, but the remaining doses exhibited a dose-dependent increase in incidence and severity of fatty liver. At 120 mg/kg/day, all dogs had microsteatosis grade 4 or grade 5.

Gene expression changes indicate toxicity already at early timepoints

There are several examples indicating an earlier detection of toxicological events by transcript profiling which precede pathology. In a comparative study, the hepatotoxic substance *coumarin* and the putatively non-hepatotoxic *6-methylcoumarin* were dosed at 200 mg/kg and mRNA levels assessed after 6, respectively 24 h. After 6 h of treatment, hepatocellular hypertrophy was observed in rats treated with coumarin, but no test item related histopathological changes were noted with 6-methylcoumarin. The toxicogenomic approach, however identified already 4 out of 5 animals treated with 6-methylcoumarin as abnormal (2 cholestatic, 2 direct acting, 1 control). After 24 h, 4 out of 5 6-methylcoumarin-treated animals were categorized as showing a direct reaction, while 1 was identified as control. All liver profiles from rats treated with coumarin were correctly identified as toxic (Cholestatic after 6 h, Direct acting after 24 h). At the 24-h time point, lymphocytic infiltration and single cell necrosis of hepatocytes were seen with both compounds, but with a clearly reduced incidence after treatment with 6-methylcoumarin. With coumarin, all 5 animals had lymphocytic infiltration (mean grade 3) and 4 animals had single cell necrosis (mean grade 1.6). With methylcoumarin 4 rats showed lymphocytic infiltration (mean grade 1) and only 1 animal had single cell necrosis (grade 1).

Tacrine hepatotoxicity was investigated at the transcriptional level and compared with serum chemistry and histopathology changes. The acute dose effects were studied after 6 and 24 h. Bioinformatic assessment of the 6-h transcript profiles flagged 4 out of 5 animals as potentially hepatotoxic (cholestasis). After 24 h, the transcriptional effect was smaller and therefore the profiles could not be distinguished from controls. After 6 h, no pathological changes were observed. After 24 h, there was a minimal reduction in glycogen deposition noted (mean grade of glycogen deposition in controls was 3; mean grade in treated animals was 2.2). Previously reported fatty change was not observed in this study. Serum changes after 6 h included increases in glucose, 5-nucleotidase (5′-NT), total protein, albumin, α1-globulin, β-gobulin. Serum changes after 24 h were increases in bile acids, γ-glutamyltransferase (GGT), alanine aminotransferase (AST), and aspartate aminotransferase (ALT).

Discussion

Rats were treated with a variety of vehicles, hepatotoxic or non-hepatotoxic compounds in order to create a reference database. We focused on hepatotoxicity since the liver is a main target for toxic reactions. Transcript profiles from individual rats were allocated to specific categories using histopathology and clinical chemistry as well as published data in order to provide sound labels (e.g., cholestatic) required for the subsequent "supervised" analysis. The SVM analysis produced gene-based binary classifiers for those predefined categories (steatosis, cholestasis, direct acting, peroxisomal proliferation or nontoxic/control). In the applied one-versus-one (OVO) training scheme, a compound-based external CV was combined with RFE in order to create a model from which we can expect a good generalization power. We published previously a one-versus-all (OVA) model on virtually the same dataset indicating that the model has indeed a good generalization power as assessed by testing transcript profiles from independent experiments (Steiner et al., 2004). With the OVO approach, more SVMs have to be trained since all pairwise comparisons between classes must be accounted for. However, the number of transcript profiles on which the models are based is smaller compared to the OVA method where one has always to use all samples that are available for training. This leads to performance gains in the model building phase. Also, we observe that a smaller number of genes is required for each binary classification, since separating two distinct classes is usually a simpler task compared to discriminating between one class and all other categories. Another advantage of the OVO approach is that in a pair wise comparison of single categories (e.g., cholestasis and steatosis), both categories consist of roughly the same number of expression profiles. This is expected to contribute to a higher sensitivity with respect to predicting the smaller groups (individual toxicity categories were considerably smaller than the vehicle control category). In total, less genes were used for classification with the OVO approach, an advantage that could be useful when developing a smaller higher-throughput assay.

Transcript profiling in combination with SVM analysis was successful in discriminating two 5-HT_6 receptor antag-

onists. Although they share the same pharmacological target and have therefore overlapping gene expression changes related to pharmacology, the toxicity-related genes allowed a successful "blinded" prediction which gene expression profiles belonged to controls or to the non-hepatotoxic compound Rx66 and which profiles indicated treatment with the steatotic compound Rx65. Interpretation of individual gene changes would be beyond the focus of this publication, but a gene-based mechanistic, rather than predictive, interpretation of transcriptional differences between these two compounds is published (Suter et al., 2003).

Identification and prediction of hepatotoxic liabilities delivered also very interesting results in a comparative analysis of 3 antidiabetic compounds. Transcriptional effects of these 3 compounds were studied after treatment for 1 or 5 days. Histopathological assessment of rat livers in several studies revealed microvesicular steatosis in rat livers treated with Rx08 and Rx09, but not with Rx10. Liver gene expression analysis was performed in a blinded manner by the previously described SVM. Whereas Rx08 and Rx09 were successfully identified as steatotic, the prediction of a steatotic liability of Rx10 was in evident contrast to pathology. Toxicogenomics was in this case used as a hypothesis generation tool for further investigations of this compound. A first measure was nile red accumulation in primary rat hepatocytes to determine the intracellular lipid content. Interestingly, all 3 compounds caused a dose-dependent increase in lipid content. This rather general finding was specified and confirmed by measurements of triglyceride accumulation in hepatocytes. Steatosis can be caused by several events like decreased production of apolipoprotein, oversupply of free fatty acids or decreased fatty acid oxidation (Treinen–Moslen, 2001). In this context, the following gene changes observed with Rx08, Rx09 and Rx10 support the prediction of a steatotic liability: apolipoprotein A1, apolipoprotein B editing complex 1, acetyl-coenzyme a acyltransferase 1, mitochondrial acyl-coa thioesterase 1, acyl-coenzyme a dehydrogenase, fatty acid binding protein 7, carnitine o-octanoyltransferase, fatty acid translocase, phospholipase A2. Other gene expression changes linked to energy metabolism included: phosphoglycerate mutase 2, malate dehydrogenase 1, glyceraldehyde-3-phosphate dehydrogenase, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and glucose-6-phosphatase, transport protein 1.

Carnitine-sensitive β-oxidation was measured in hepatocytes and indicated a decreased rate of β-oxidation upon treatment with the 3 compounds Rx08, Rx09 and Rx10. The effect with Rx09 and Rx10 was more pronounced than the reduction in β-oxidation induced by Rx08, which was restricted to a relatively high concentration (300 μM). During the short duration of the incubation period (2 h) and in the presence of 2% albumin in these experiments no increased LDH release was observed, not even at this high concentration. Together, these in vitro experiments support the flag for steatosis raised by toxicogenomics for the putatively not hepatotoxic compound Rx10. Interestingly, a 14-day dog study provided clear evidence for the predicted hepatotoxicity of Rx10 as both necrosis and steatosis were observed in the dog liver. Although this is an impressive example for cross-species extrapolation based on transcript profiling, it remains to be seen how often a potentially slightly sub-toxic dose in one species can already be indicative for toxicity. Since different species have different ADME properties (Yamaguchi et al., 2001), this approach is not expected to always succeed, but in some cases, indicative information can be found. This is most likely the case if toxicity is just slightly below detection limits of routine methods. Therefore, we investigated cases where the toxicogenomic approach could provide a gain in sensitivity. The comparative study with coumarin and the supposedly non-hepatotoxic 6-methylcoumarin (Lake, 1999) highlighted a temporal gain in detecting toxicity using toxicogenomics. Coumarin was predicted to be hepatotoxic after 6 and 24 h by the SVM, which was in line with the histopathological assessment. Although the identification as toxic was correct, the predicted mechanism cholestasis is questionable since serum changes did not support this assumption. After 24 h, necrosis and lymphocyte infiltration were observed, which are typical findings for direct acting compounds. With 6-methylcoumarin, no treatment-related changes were noted after 6 h, but 4 out of 5 animals were considered as different from controls by the SVM. The suspected hepatotoxicity could be confirmed by histopathology after a 24-h period, therefore supporting the hypothesis that transcriptional changes can be used to detect undesired effects at an earlier time.

The dose limiting factor for tacrine is liver enzyme elevation observed in Alzheimer's patients (Hammel et al., 1990). No changes were observed in rats 6 h after dosing with tacrine. After 24 h, there was a slight reduction in liver glycogen content and transaminase levels were elevated as previously reported. The toxicogenomic assessment of a potential cholestatic liability of tacrine was supported by increased serum levels of bile acids and GGT. However, since both AST and ALT were increased, this indicates mixed hepatotoxicity and not pure cholestasis. The toxicogenomic classification detected the liver toxicity after 6 h, but failed to do so after 24 h. This indicates that gene expression changes can precede clinical chemistry changes and implies the need for time-course studies for a thorough toxicogenomic assessment. Temporal differences in toxicant induced gene expression changes have been reported before (Heijne et al., 2004; Ruepp et al., 2002). Drugs have pharmacological as well as toxicological effects which might affect gene expression. These two effects can, but need not be related.

The promising use of a database of liver transcript profiles to identify potential safety issues has been described by several research groups. An early example described how a 15-hepatotoxin gene expression database was successfully used to identify the mechanism of toxicity associated with a development compound (Waring et al., 2002). However, a

limiting factor in the ability to classify novel compounds to toxic mechanisms is clearly the relatively small number of investigated compounds. Only recently, larger databases and their use in compound classification have been described (Kier et al., 2004; McMillian et al., 2004; Steiner et al., 2004). These studies varied in design, number of compounds investigated, and bioinformatics processing of the data, but all indicated the potential of toxicogenomics in predictive risk assessment.

Our results indicate that characteristic gene expression changes are associated with distinct classes of toxicants. In general, we observed good concordance of gene expression changes with histopathological findings assessed by light microscopy. However, results with 6-methylcoumarin and tacrine suggest that gene expression changes are already indicative of toxic liabilities when standard parameters do not yet detect toxicity. A similar finding was reported in a study where a good concordance of gene expression changes with histopathological grade was observed, but indicative gene changes were already noted at low doses in the absence of obvious microscopic alterations (Hamadeh et al., 2002b).

The current model assigns all transcript profiles to a specific category, implying that they fit exactly into one class. However, in reality substances will often cause mixed toxicities. We aimed to allocate substances to the best fitting class, knowing the limitations due to the potential overlap of effects. It has to be kept in mind that the compound database is still limited in size and we do not have the data for further endpoints (e.g., fibrosis). It is also unknown to which extent the present compound selection is relevant for the complete compendium of hepatotoxic substances.

In summary, we demonstrated how toxicogenomics can be applied to discriminate compounds of the same pharmacological class and flag substances for potential hepatotoxicity. A promise of this approach lies in better prioritization of compounds in development and earlier identification of potential "show-stopping" toxicities.

Acknowledgments

We would like to thank M Haiker, A Huber, N Flint, S Romer, K Rupp, K Schad, C Zihlmann, the General Toxicology group and the bioinformatics group for their excellent support.

References

Bartosiewicz, M., Penn, S., Buckpitt, A., 2001. Applications of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium chloride, benzo(a)pyrene, and trichloroethylene. Environ. Health Perspect. 109, 71–74.

Baumgartner, C., Bohm, C., Baumgartner, D., Marini, G., Weinberger, K., Olgemoller, B., Liebl, B., Roscher, A.A., 2004. Supervised machine learning techniques for the classification of metabolic disorders in newborns. Bioinformatics.

Berry, M.N., Friend, D.S., 1969. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 43, 506–520.

Boser, B., IM, G., VN, V., 1992. A training algorithm for optimal margin classifiers. Proceedings of the 5th Annual International Conference on Computational Learning Theory. ACM Press, Pittsburgh, PA, pp. 144–152.

Bulera, S.J., Eddy, S.M., Ferguson, E., Jatkoe, T.A., Reindel, J.F., Bleavins, M.R., De La Iglesia, F.A., 2001. RNA expression in the early characterization of hepatotoxicants in Wistar rats by high-density DNA microarrays. Hepatology 33, 1239–1258.

Bullinger, L., Dohner, K., Bair, E., Frohling, S., Schlenk, R.F., Tibshirani, R., Dohner, H., Pollack, J.R., 2004. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N. Engl. J. Med. 350, 1605–1616.

Chang, C.C., Lin, C.J., 2001. LIBSVM: a library for support vector machines. Software available at http://www.csie.ntu.edu.tw/~cjlin/libsvm.

Cristianini, N., Shawe-Taylor, J., 2000. An Introduction to Support Vector Machines and Other Kernel-Based Learning Methods. Cambridge Univ. Press, Cambridge, UK.

Fowler, S.D., Brown, W.J., Warfel, J., Greenspan, P., 1987. Use of nile red for the rapid in situ quantitation of lipids on thin-layer chromatograms. J. Lipid Res. 28, 1225–1232.

Goldlin, C.R., Boelsterli, U.A., 1991. Reactive oxygen species and non-peroxidative mechanisms of cocaine-induced cytotoxicity in rat hepatocyte cultures. Toxicology 69, 79–91.

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S., 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531–537.

Greenspan, P., Mayer, E.P., Fowler, S.D., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965–973.

Guyon, I., Weston, J., Barnhill, S., Vapnik, V., 2002. Gene selection for cancer classification using support vector machines. Mach. learn., 389–442.

Hamadeh, H.K., Bushel, P.R., Jayadev, S., DiSorbo, O., Bennett, L., Li, L., Tennant, R., Stoll, R., Barrett, J.C., Paules, R.S., Blanchard, K., Afshari, C.A., 2002a. Prediction of compound signature using high density gene expression profiling. Toxicol. Sci. 67, 232–240.

Hamadeh, H.K., Knight, B.L., Haugen, A.C., Sieber, S., Amin, R.P., Bushel, P.R., Stoll, R., Blanchard, K., Jayadev, S., Tennant, R.W., Cunningham, M.L., Afshari, C.A., Paules, R.S., 2002b. Methapyrilene toxicity: anchorage of pathologic observations to gene expression alterations. Toxicol. Pathol. 30, 470–482.

Hammel, P., Larrey, D., Bernuau, J., Kalafat, M., Freneaux, E., Babany, G., Degott, C., Feldmann, G., Pessayre, D., Benhamou, J.P., 1990. Acute hepatitis after tetrahydroaminoacridine administration for Alzheimer's disease. J. Clin. Gastroenterol. 12, 329–331.

Heijne, W.H., Slitt, A.L., van Bladeren, P.J., Groten, J.P., Klaassen, C.D., Stierum, R.H., van Ommen, B., 2004. Bromobenzene-induced hepatotoxicity at the transcriptome level. Toxicol. Sci. 79, 411–422.

Jaeschke, H., Gores, G.J., Cederbaum, A.I., Hinson, J.A., Pessayre, D., Lemasters, J.J., 2002. Mechanisms of hepatotoxicity. Toxicol. Sci. 65, 166–176.

Kier, L.D., Neft, R., Tang, L., Suizu, R., Cook, T., Onsurez, K., Tiegler, K., Sakai, Y., Ortiz, M., Nolan, T., Sankar, U., Li, A.P., 2004. Applications of microarrays with toxicologically relevant genes (tox genes) for the evaluation of chemical toxicants in Sprague Dawley rats in vivo and human hepatocytes in vitro. Mutat. Res. 549, 101–113.

Lake, B.G., 1999. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food Chem. Toxicol. 37, 423–453.

Lee, C.K., Brown, B.G., Reed, E.A., Hejtmancik, M., Mosberg, A.T., Doolittle, D.J., Hayes, A.W., 1992. DNA adduct formation in mice

following dermal application of smoke condensates from cigarettes that burn or heat tobacco. Environ. Mol. Mutagenesis 20, 313–319.

McClelland, M., Sanderson, K.E., Spleth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., All, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., Wilson, R.K., 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature 413, 852–856.

McMillian, M., Nie, A.Y., Parker, J.B., Leone, A., Kemmerer, M., Bryant, S., Herlich, J., Yieh, L., Bittner, A., Liu, X., Wan, J., Johnson, M.D., 2004. Inverse gene expression patterns for macrophage activating hepatotoxicants and peroxisome proliferators in rat liver. Biochem. Pharmacol. 67, 2141–2165.

Pessayre, D., Mansouri, A., Haouzi, D., Fromenty, B., 1999. Hepatotoxicity due to mitochondrial dysfunction. Cell Biol. Toxicol. 15, 367–373.

Roberts, R.A., James, N.H., Hasmall, S.C., Holden, P.R., Lambe, K., Macdonald, N., West, D., Woodyatt, N.J., Whitcome, D., 2000. Apoptosis and proliferation in nongenotoxic carcinogenesis: species differences and role of PPARalpha. Toxicol. Lett. 112–113, 49–57.

Ross, D.T., Scherf, U., Eisen, M.B., Perou, C.M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S.S., Van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J.C., Lashkari, D., Shalon, D., Myers, T.G., Weinstein, J.N., Botstein, D., Brown, P.O., 2000. Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet. 24, 227–235.

Ruepp, S.U., Tonge, R.P., Shaw, J., Wallis, N., Pognan, F., 2002. Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. Toxicol. Sci. 65, 135–150.

Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T.G., Andrews, D.T., Scudiero, D.A., Eisen, M.B., Sausville, E.A., Pommier, Y., Botstein, D., Brown, P.O., Weinstein, J.N., 2000. A gene expression database for the molecular pharmacology of cancer. Nat. Genet. 24, 236–244.

Scholkopf, B., Smola, A., 2002. Learning with Kernels. MIT Press, Cambridge, MA.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C.M., Lonning, P.E., Brown, P.O., Borresen-Dale, A.L., Botstein, D., 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. U.S.A. 100, 8418–8423.

Steiner, G., Suter, L., Boess, F., Gasser, R., de Vera, M.C., Albertini, S., Ruepp, S., 2004. Discriminating different classes of toxicants by transcript profiling. Environ. Health Perspect. 1236–1248.

Suter, L., Haiker, M., De Vera, M.C., Albertini, S., 2003. Effect of two 5-HT(6) receptor antagonists on the rat liver: a molecular approach. Pharmacogenomics J. 3, 320–334.

Thomas, R.S., Rank, D.R., Penn, S.G., Zastrow, G.M., Hayes, K.R., Pande, K., Glover, E., Silander, T., Craven, M.W., Reddy, J.K., Jovanovich, S.B., Bradfield, C.A., 2001. Identification of toxicologically predictive gene sets using cDNA microarrays. Mol. Pharmacol. 60, 1189–1194.

Treinen-Moslen, M., 2001. Casarett and Doull's Toxicology: the Basic Science of Poisons, 6th ed. In: Klaasen, C.D. (Ed.). McGraw-Hill, New York, NY.

Vapnik, V., 1998. Statistical Learning Theory. Wiley, New York.

Velayudham, L.S., Farrell, G.C., 2003. Drug-induced cholestasis. Expert Opin. Drug Saf. 2, 287–304.

Waring, J.F., Ulrich, R.G., 2000. The impact of genomics-based technologies on drug safety evaluation. Annu. Rev. Pharmacol. Toxicol. 40, 335–352.

Waring, J.F., Jolly, R.A., Ciurlionis, R., Lum, P.Y., Praestgaard, J.T., Morfitt, D.C., Buratto, B., Roberts, C., Schadt, E., Ulrich, R.G., 2001. Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. Toxicol. Appl. Pharmacol. 175, 28–42.

Waring, J.F., Gum, R., Morfitt, D., Jolly, R.A., Ciurlionis, R., Heindel, M., Gallenberg, L., Buratto, B., Ulrich, R.G., 2002. Identifying toxic mechanisms using DNA microarrays: evidence that an experimental inhibitor of cell adhesion molecule expression signals through the aryl hydrocarbon nuclear receptor. Toxicology 181–182, 537–550.

Yamaguchi, Y., Touchi, A., Baba, T., Matsubara, T., 2001. Species differences in beta-oxidative metabolism of a thromboxane A2-receptor antagonist [(+)-S-145] in rat, dog and monkey. Xenobiotica 31, 125–133.